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(71) Applicant *(for all designated States except US)*: **PARKER HUGHES INSTITUTE** [US/US]; 2665 Long Lake Road, St. Paul, MN 55113 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **UCKUN, Fatih, M.** [US/US]; 12590 Ethan Avenue North, White Bear Lake, MN 55110 (US). **ZHU, De-Min** [US/US]; 8386 Pleasantview Drive, Mounds View, MN 55112 (US).

(74) Agent: **DAIGNAULT, Ronald, A.**; Merchant & Gould P.C., P.O. Box 2903, Minneapolis, MN 55402-0903 (US).

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(54) Title: CALPAIN INHIBITORS IN CANCER TREATMENT

(57) Abstract: Embodiments of the present invention provide methods for inhibiting tumor cell growth, or treating cancer, in a subject through the administration of a calpain inhibitor or inhibitors. Methods for inhibiting inflammatory disease cells as well as other calpain expresing cells in a subject with a calpain inhibitor or inhibitors are also within the present invention. Furthermore, the present invention relates to inducing cytotoxicity or apoptosis in cells by administering a cytotoxic or an apoptotic dose of a calpain inhibitor or inhibitors to the cell. Finally, the present invention relates to the administration of calpain expressing vectors to tumor cells so as to inhibit the tumor cells growth.

CALPAIN INHIBITORS IN CANCER TREATMENT

Field of the Invention

This invention relates to inhibition of calpain for treatment of cancer and more particularly to the induction of apoptosis in human cancer cells by inhibitors of calpain.

Background of the Invention

Apoptosis, also referred to as programmed cell death, is a form of cell death characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation and DNA fragmentation. Willie (1980) Int. Rev. Cytol., 68: 251-306. Many genes are involved in the apoptotic process. In general, the products of these genes are classified as either inducers or inhibitors of apoptosis. Apoptotic signaling pathways involve substantial interactions between the products of the apoptotic inducers and the apoptotic inhibitors. Oltvai et al. (1993) Cell, 80: 293-299.

The identification and development of new potent drugs that can trigger apoptosis is a focal point of cancer treatment and biomedical research, this is especially true in the fields of translational leukemia and lymphoma research.

Recently, the inventors of the present invention discovered that calcium mobilizers can induce apoptosis in acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma (NHL) in a protein kinase C (PKC)-dependent fashion. Zhu et al. (1998) Clin. Cancer Res., 4: 2967-2976; Zhu et al. (1999) Clin. Cancer Res., 5: 355-360. Thus, it was of interest in the art to further investigate other possible calcium dependent apoptotic pathways.

Calpains, a group of calcium activated cytosolic proteases, have been implicated in calcium dependent death (Squier et al. (1997) J Immonol., 158: 3690-3697; Villa et al. (1998) J Cell Science, 111: 713-722; Nath et al. (1996) Neutropharmcol. Neurotoxicol., 8:249-255; Nath et al. (1996) Biochem J, 319: 683-690; Gressner et al. (1997) Biochem. Biophys. Res. Commun., 231: 457-462; McGinnis et al. (1998) J Biol. Chem., 273: 19993-20000; Jordán et al. (1997) J Neurochem., 68: 1612-1621; Edelstein et al. (1996) Ren. Fail., 18: 501-511;

Sarin et al. (1994) J Immunol., 153: 862-872; Squir et al. (1994) J Cell Physiol., 159: 229-237) as well as in other normal physiologic and pathologic conditions. Beckerle et al. (1987) Cell, 51: 569-577. Additionally, calpain inhibitors are generally considered inhibitors of calpain-dependent apoptosis. Nath et al. (1996) Neutropharmcol. Neurotoxicol., 8:249-255; Nath et al. (1996) Biochem J, 319: 683-690; Gressner et al. (1997) Biochem. Biophys. Res. Commun., 231: 457-462; Sarin et al. (1994) J Immunol., 153: 862-872; Wang et al. (1994) TiPS, 15: 412-419; Wang et al. (1997) Adv. Pharmacol., 37: 117-152. Against this backdrop the present invention has been developed.

Summary of the Invention

The present invention involves the surprising and unexpected discovery that calpain inhibitors and methods of use for calpain inhibitors trigger caspase dependent apoptosis in cancer cells and in cells having aberrant levels of calpain.

One aspect of the present invention is a method for inhibiting the growth of tumor cells in a subject by administering to the subject a calpain inhibitor. The method can include inducing the tumor cells to become apoptotic. Preferable types of tumor cells in the context of the present invention include acute lymphoblastic leukemia and non-Hodgkin's lymphoma.

Another aspect of the invention is a method of treating cancer cells in a subject by administering a therapeutically effective amount of calpain inhibitor to the subject. The cancer cells can be solid cancer cells, for example squamous cell carcinoma, but are more preferably white blood cells, for example acute lymphoblastic leukemia and non-Hodgkin's lymphoma.

Another aspect of the invention is a method for inhibiting inflammatory disease in a subject by administering to the subject a calpain inhibitor. The inflammatory disease can be rheumatoid arthritis.

Another aspect of the invention is a method for inducing cytotoxicity in a cell by administering a cytotoxic dose of calpain inhibitor to the cell.

Another aspect of the invention is a pharmaceutical composition having a substantially purified calpain inhibitor and a pharaceutically acceptable carrier.

Another aspect of the invention is a method for inhibiting growth of a tumor cell by making a recombinant vector that expresses a calpain inhibitor and administering the recombinant vector to the tumor cell.

Finally, another aspect of the invention is a method for inducing apoptosis in a cell by expressing a heterologous nucleic acid sequence encoding the calpain inhibitory peptide in a host cell having enhanced calpain activity as compared to control host cells.

Brief Description of the Drawings

Figure **1** is a microscopic image of tumor cells stained with anti-calpain monoclonal antibodies and FITC-labeled goat anti-mouse IgG.

Figure **2** shows a representative FACS-correlated display of CPI-2 treated human ALL and NHL cells. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence.

Figure **3** illustrates that myeloid leukemia cells are resistant to apoptosis as induced by CPI-2 treatment. The percentage of apoptotic cells is the sum of the percentages of the cells at early and advanced apoptotic stages.

Figure **4** illustrates that solid tumor cell lines are generally resistant to apoptosis as induced by CPI-2 treatment. Only the SQ-20B cell line showed CPI-2 induced apoptosis. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence.

Figure 5 shows a representative FACS-correlated display of CPI-2 treated DT-40 wild type, DT-40 LYN deficient and DT-40 BTK deficient cells. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence.

Figure 6 shows a representative FACS-correlated display of CPI-2 treated human MOLT-3 and SQ-20B cells in the presence and absence of caspase inhibitor I.

Detailed Description of the Invention

Definitions:

The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

The term "apoptosis" refers to the genetically programmed process of cell death characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation and DNA fragmentation. Wyllie (1990) Int. Rev. Cytol., 68: 251-306. Apoptotic regulatory genes include the p53 tumor suppresser gene, the Bcl-2 gene family, and the caspase family of genes.

The term "cell line", "host cell" or "host cells" refers to cells established in *ex vivo* culture. It is a characteristic of host cells discussed in the present disclosure that they be capable of expressing calpain and calpain inhibitors. Examples of suitable host cells useful for aspects of the present invention include insect and mammalian cells. Specific examples of such cells include SF9 insect cells (Summers and Smith (1987) Texas Agriculture Experiment Station Bulletin, 1555), human primary embyonal kidney cells (293 cells), Chinese hamster ovary (CHO) cells (Puck et al. (1958) Proc. Natl. Acad. Sci. USA, 60, 1275-1281), human cervical carcinoma cells (HELA) (ATCC CCL 2), a multi-drug and radiation resistant human squamous cell carcinoma (SQ-20B), human breast cancer cells (BT-20), glioblastoma cells (U373), CML cells (K-562), a highly radiation-resistant MILL-AF4 fusion transcript positive t(4;11) pre-pre B ALL cell line (RS4;11), a multidrug resistant BCR-ABL fusion transcript positive t(9;22) pro-B ALL cell line (ALL-1); a highly radiation-resistant and p53 deficient Burkitt's lymphoma cell line (RAMOS) (Myers et al. (1995) Proc. Natl. Acad. Sci. USA, 92: 9575-9579); a Burkitt's leukemia/lymphoma cell line (DAUDI); T-lineage ALL/NHL cells (JURKAT and MOLT-3); a pre-B ALL cell line (NALM-6); a LYN deficient clone and BTK deficient clone of DT-40 cells (Uckun et al. (1996) Science, 273: 1096-1100), etc.

The term "calpain inhibitor" refers to chemical compositions, polypeptides, polynucleotides, etc. that inhibit the enzymatic activity of calpain

family members. One example of a calpain inhibitor useful in the present invention is calpain inhibitor II (N-Ac-Leu-Leu-Met) (CPI-2). CPI-2 may be purchased from Calbiochem, La Jolla, California.

The terms "cancer" and "cancerous" refer to or describe the physiologic condition in mammals that is typically characterized by the loss of responsiveness to normal growth controls. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma and leukemia. Cancers can originate from among other things epithelial cells like a carcinoma, or can originate from mesenchymal cells like cancers of the blood cells. Examples of carcinomas include but are not limited to squamous cell, adenocarcinoma and melanoma and examples of cancers of the blood cells are leukemia and lymphoma. For purposes of this disclosure, solid tumors are tumors that are not of blood cell origin.

As used herein, "cDNA" refers to recombinant DNA formed from the mRNA of the target protein, in this case a calpain inhibitor mRNA. cDNA molecules can be inserted into vectors that favor their expression in host cells.

As used herein, "control host cell" refers to a cell that has been cultured in parallel with a cell treated under the specified experimental condition but unlike the treated cell, the host cell has not undergone the specified experimental condition. Control cells represent a baseline from which comparisons are made.

The term "inflammatory disease" or "disorder" refers to a fundamental pathogenic process consisting of a dynamic complex of cytologic and histologic reactions that occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by physical, chemical, or biologic agent. Examples of inflammatory disease within the context of the present invention include, rheumatoid arthritis, osteoarthritis, etc.

The term "nucleic acid sequence" refers to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The deoxyribonucleotide sequence thus codes for the amino acid sequence.

As used herein, "pharmaceutically acceptable salt thereof" includes an acid addition salt or a base salt.

As used herein, "pharmaceutically acceptable carrier" includes any material which, when combined with a compound of the invention, allows the compound to retain biological activity, such as the ability to induce apoptosis of leukemia or breast tumor cells, and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsions, and various types of wetting agents. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, Chapter 43, 14th Ed., Mack Publishing Co., Easton, PA).

The term "polynucleotide" refers to a linear sequence of nucleotides. The nucleotides are either a linear sequence of polyribonucleotides or polydeoxyribonucleotides, or a mixture of both. Examples of polynucleotides in the context of the present invention include - single and double stranded DNA, single and double stranded RNA, and hybrid molecules that have both mixtures of single and double stranded DNA and RNA. Further, the polynucleotides of the present invention may have one or more modified nucleotides.

The term "subject" in the context of this invention means a mammal, *i.e.*, any class of higher vertebrates that nourish their young with milk secreted by mammary glands.

The term "treating" or "treatment" in the context of this invention means the prevention or reduction of severity symptoms or effect of a pathological condition, including prolonging life expectancy. In the context of cancer therapy, treatment includes prevention, of tumor growth, reduction of tumor size, enhanced tumor cell death, and increased apoptosis.

The term "tumor cell" within the context of the present invention is used synonymously with cancer cell and means a cell that has lost, in some manner, its ability to respond to normal growth signals, i.e., is undergoing abnormally regulated growth.

The term "vector", "extra-chromosomal vector" or "expression vector" refers to a first piece of DNA, usually double-stranded, which may have inserted into it a second piece of DNA, for example a piece of foreign DNA like the cDNA

of CPI-2. Foreign DNA is defined as heterologous DNA, which is DNA that may or may not be naturally found in the host cell and includes additional copies of nucleic acid sequences naturally present in the host genome. The vector transports the foreign DNA into a suitable host cell. Once in the host cell the vector may be capable of integrating into the host cell chromosomes. The vector may also contain the necessary elements to select cells containing the integrated DNA as well as elements to promote transcription of mRNA from the transfected DNA. Examples of vectors within the scope of the present invention include, but are not limited to, plasmids, bacteriophages, cosmids, retroviruses, and artificial chromosomes.

Finally, in order to facilitate the understanding of the present invention the following list of commonly used abbreviations is provided: Acute Lymphoblastic Leukemia (ALL); non-Hodgkin's Lymphoma (NHL); Bruton's tyrosine kinase (BTK); calpain inhibitor II (CPI-2); Propidium Iodide (PI).

Modes for Carrying out the Invention:

The present invention includes novel and unexpected methods of use for calpain inhibitors in the selective triggering of caspase dependent apoptosis in cancer cells having aberrant levels of calpain as well as in non-cancerous cells having aberrant levels of calpain. Calpain inhibitors, for example CPI-2, may be useful agents in the treatment of cancer, for example by inhibitting tumor growth or by killing tumor cells.

Preferable cancers for treatment with embodiments of the present invention include ALL and NHL. Calpain inhibitors may also be of use in the treatment of inflammatory disease or any other disease state where the cells show aberrant calpain expression.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook et al. (1989) Molecular cloning: A Laboratory Manual), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991 Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, 3d., (1990) Academic

Press, Inc.), *PCR Protocols: A Guide to Methods and Applications* (Innis et al. (1990) Academic Press, San Diego, CA), Culture of Animal Cells: A Manual of Basic Technique, 2nd ed. (R.I. Freshney (1987) Liss, Inc., New York, NY), *and Gene Transfer and Expression Protocols*, pp 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

Cytotoxic Compounds

The calpain inhibitors of the invention are effective cytotoxic agents, for example, against tumor cells such as acute lymphoblastic leukemia and non-Hodgkin's lymphoma. In the methods of the invention, the cytotoxic effects of calpain inhibitors are achieved by treating, such as tumor cells, with micromolar amounts of the inhibitory compound. As shown in the Examples below, when apoptosis is measured, incubation of cells with a calpain inhibitor results in at least about 10% or greater of the cells becoming apoptotic. By way of example, a particularly useful anti-tumor agent is CPI-2 as shown in the Examples below. Other calpain inhibitors may be known to those of skill in the art.

The present invention also includes polynucleotide encoding calpain inhibitors including fragments, analogs and derivatives of CPI-2 as cytotoxic compounds. A fragment, analog or derivative may be made by mutagenesis techniques or other methods known to the art. Additionally, the polynucleotide fragments, analogs and derivatives may include substitutions, deletions or additions that involve one or more nucleotides.

Another embodiment of the present invention are polynucleotides that are at least 75% identical to the polynucleotide sequence for CPI-2 as used as cytotoxic compounds. Further, preferred embodiments are between 80 and 95% identical to the CPI-2 cDNA and highly preferred embodiments are between 95 and 99% identical.

Tumor Treatment

The calpain inhibitors of the invention can also be used in methods of tumor treatment, for example, by administering to a subject a calpain inhibitor in order to

achieve an inhibition of tumor cell growth, a killing of tumor cells, induced apoptosis, and/or increased patient survival time.

The present invention also includes polynucleotide fragments, analogs and derivatives of CPI-2 as used in tumor treatment. A fragment, analog or derivative may be made by mutagenesis techniques or other methods known to the art. Additionally, the polynucleotide fragments, analogs and derivatives may include substitutions, deletions or additions that involve one or more nucleotides.

Another embodiment of the present invention are polynucleotides that are at least 75% identical to the polynucleotide sequence for CPI-2 as used in tumor treatment. Further, preferred embodiments are between 80 and 95% identical to the CPI-2 cDNA and highly preferred embodiments are between 95 and 99% identical.

The calpain inhibitors of the invention are suitable for use in mammals. As used herein, "mammals" means any class of higher vertebrates that nourish their young with milk secreted by mammary glands, including, for example, humans, rabbits, and monkeys.

Administration Methods

The calpain inhibitors of the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, including a human patient, in a variety of forms adapted to the chosen route of administration. The compounds are preferably administered in combination with a pharmaceutically acceptable carrier, and may be combined with or conjugated to specific delivery agents, including targeting antibodies and/or cytokines.

The calpain inhibitors can be administered by known techniques, such as orally, parentally (including subcutaneous injection, intravenous, intramuscular, intrasternal or infusion techniques), by inhalation spray, topically, by absorption through a mucous membrane, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants or vehicles. Pharmaceutical compositions of the invention can be in the form of suspensions or tablets suitable for oral administration, nasal sprays, creams, sterile injectable

preparations, such as sterile injectable aqueous or oleagenous suspensions or suppositories.

For oral administration as a suspension, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The compositions can contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners or flavoring agents. As immediate release tablets, the compositions can contain microcrystalline cellulose, starch, magnesium stearate and lactose or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

For administration by inhalation or aerosol, the compositions can be prepared according to techniques well-known in the art of pharmaceutical formulation. The compositions can be prepared as solutions in saline, using benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons or other solubilizing or dispersing agents known in the art.

For administration as injectable solutions or suspensions, the compositions can be formulated according to techniques well-known in the art, using suitable dispersing or wetting and suspending agents, such as sterile oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

For rectal administration as suppositories, the compositions can be prepared by mixing with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ambient temperatures, but liquefy or dissolve in the rectal cavity to release the drug.

Preferred administration routes include orally, parenterally, as well as intravenous, intramuscular or subcutaneous routes. More preferably, the compounds of the present invention are administered parenterally, i.e., intravenously or intraperitoneally, by infusion or injection. In one embodiment of the invention, the compounds may be administered directly to a tumor by tumor injection; or by systemic delivery by intravenous injection.

Solutions or suspensions of the compounds can be prepared in water, isotonic saline (PBS) and optionally mixed with a nontoxic surfactant. Dispersions may also be prepared in glycerol, liquid polyethylene, glycols, DNA, vegetable oils, triacetin

and mixtures thereof. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms. The pharmaceutical dosage form suitable for injection or infusion use can include sterile, aqueous solutions or dispersions or sterile powders comprising an active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol such as glycerol, propylene glycol, or liquid polyethylene glycols and the like, vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size, in the case of dispersion, or by the use of nontoxic surfactants. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, buffers, or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the inclusion in the composition of agents delaying absorption--for example, aluminum monosterate hydrogels and gelatin.

Sterile injectable solutions are prepared by incorporating the compounds in the required amount in the appropriate solvent with various other ingredients as enumerated above and, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

Vectors and host cells:

In one aspect of the present invention, novel polynucleotides substantially similar to the CPI-2 polynucleotide sequence are subcloned into an extra-chromosomal vector. The subcloned polynucleotide(s) may be joined to a vector

having a cis-acting or regulatory element for increased propagation in a host cell (note that the trans-acting factors involved are supplied to the host, supplied by a second vector or supplied by the vector itself upon introduction into the host). This aspect of the invention allows for the *in vivo* and *in vitro* expression of the CPI-2 polynucleotide, thus permitting an analysis of cathepsin activity and function. Several vectors can be used in the context of this invention, including: PcDNA3 vector (Invitrogen), vectors having the T3 and T7 polymerase promoters, vectors having the SV40 promoter or the CMV promoter, or any other promoter that either can direct expression of a polypeptide off a polynucleotide, or that one wishes to test for the ability to direct expression of a polypeptide off a polynucleotide.

The present invention also includes polynucleotide fragments, analogs and derivatives of CPI-2 that are subcloned into extra-chromosomal vecotors. A fragment, analog or derivative may be made by mutagenesis techniques or other methods known to the art. Additionally, the polynucleotide fragments, analogs and derivatives may include substitutions, deletions or additions that involve one or more nucleotides.

Another embodiment of the present invention are polynucleotides that are at least 75% identical to the polynucleotide sequence for CPI-2 as subcloned into extra-chromosomal vectors. Further, preferred embodiments are between 80 and 95% identical to the CPI-2 cDNA and highly preferred embodiments are between 95 and 99% identical.

In a further aspect of the present invention, host cells can be genetically engineered to incorporate the polynucleotides encoding calpain inhibitors of the present invention and to express the polypeptides of the present invention. Techniques required for this aspect of the invention are well known in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, 1989) and can include calcium phosphate transfection, dextran sulfate transfection, electroporation, lipofection and viral infection (see Graham and van der Eb (1978) Virology, 52, 456-457; Chisholm et al. (1995) DNA Cloning IV: A Practical Approach, Mammalian Systems, Glover and Hanes, eds., pp 1-41; Andreason (1993) J. Tisss. Cult. Meth., 15, 56-62).

The host cells of the present invention may be of any type, including, but limited to, non-eukaryotic and eukaryotic cells. Host cells are cultured using standard tissue culture techniques in conventional media as is well known in the art. The level of expression of the CPI-2 cDNA introduced into a host cell of the invention depends on multiple factors, including gene copy number, efficiency of transcription, messenger RNA processing, stability, and translation efficiency. Accordingly, high level expression of a desired CPI-2 polypeptide according to the present invention will typically involve optimizing one or more of those factors.

EXAMPLES

The following examples are provided to illustrate the invention only, and should not be construed as limiting the scope of the invention.

Example I

Tumor Cell Lines and Culture Conditions

The experiments of the present invention were conducted on several different acute lymphoblastic leukemia, non-Hodgkin's lymphoma, myeloid leukemia and solid tumor cell lines. The acute lymphoblastic leukemia, non-Hodgkin's lymphoma and myeloid leukemia were all cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% head-inactivated fetal bovine serum (Summit Biotech, Ft. Collins, CO), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY). The solid tumor lines, HeLa and U373, were cultured in MEM supplemented with non-essential amino acids, Earl's BSS, 1 mM sodium pyruvate and 10% fetal bovine serum. The solid tumor line SQ-20B was grown in DMEM supplemented with 20% fetal bovine serum and the line PC-3 cells was cultured in Ham's F12K medium supplemented with 10% fetal bovine serum. Additionally, wild type and LYN and BTK deficient, chicken lymphoma B-cell DT-40 clones (Uckon et al. (1996) Science, 273: 1096-1100) were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% head-inactivated fetal bovine serum (Summit Biotech, Ft.

Collins, CO), 1% chicken serum (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY).

Example II

Calpain Expression in Human Tumor Cell Lines

Experiments were conducted to determine calpain expression in leukemia and solid tumor cells. Cell lines were cultured using methods well known in the art. Solid tumor cells were harvested and plated on coverslips. Suspension cells (leukemia cells) were harvested and plated onto Superfrost Plus slides (Sigma, St. Louis, MO). Plated cells were fixed in methanol at -20°C for 15 minutes. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS in the presence of 0.1% sodium citrate for 15 minutes. Non-specific binding sites were blocked with 2% bovine serum albumin (BSA) in PBS for 15 minutes. After three washes with PBS, cells were incubated with 100 μl anti-calpain monoclonal antibody (Chemicon International Inc., Temecula, CA) (1:100 dilution) at 37°C for one hour, followed by one wash with PBS. Cells were then incubated with 2.5 μg/ml FITC-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) at 37°C for 40 minutes. Cells were then washed in PBS and the coverslips mounted with Vectashield containing propidium iodide (Vector Labs). Fluorescence images of the cells were taken by a laser scanning confocal microscope (MRC 1024, Bio-Rad) and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Results:

As shown in Fig. 1, human ALL and NHL cells (ALL-1 and NALM-6) express high levels of calpain. However, only one of the four solid tumor cell lines examined, SQ-20B, expressed high levels of calpain. The other three solid tumor cell lines showed little to no calpain expression.

Example III

Calpain Inhibitor-2 Induces Apoptosis in Human ALL and NHL

Quantitative flow cytometic apoptosis detection assays and TUNEL assays were performed to determine if calpain inhibitor-2 (CPI-2) treated ALL, NHL, myeloid lymphoma cells and solid tumor cells undergo apoptosis.

Quantitative flow cytometric apoptosis detection assays were performed on ALL cells (ALL-1, RS4;11, JURKAT), NHL cells (RAMOS, DAUDI), myeloid leukemia cells (K562, HL-60, U937) and solid tumor cells (SQ20B, HeLa, U373, PC-3). Cells were treated with CPI-2 at varying concentrations for 24 hours. Just prior to the assay, one mg/ml stock solutions of MC540 and PI were filtered through a 0.22 μm filter and stored at 4°C in the dark. Each cell line was harvested, and $1x10^6$ cells suspended and stained with 5 μg/ml MC540 and 10 μg/ml propidium iodide (PI) (Uckun et al. (1996) Science, 273: 1096-1100) at 4°C in the dark for 24 hours. Stained cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) using the 488-nm excitation from an argon laser. MC540 and PI emissions were split with a 600-nm short pass diachronic mirror; a 575-nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission, and 635-nm band pass filter was used for PI emission. In each experiment approximately 10,000 cells were analyzed by FACS and the percentage of cells at early (MC540 fluorescence only) and advanced (dual MC540 plus PI fluorescence) stages of apoptosis obtained.

DNA cleavage as an indicator of apoptosis was determined using the *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling method (TUNEL assay) of Boehringer Mannheim (Cell Death Detection Kit) as described in Zhu et al. (1999) Clin. Can. Res., 5:355-360. Briefly, CPI-2 treated cells (50 or 100 μM) and control cells were harvested and resuspended in phosphate-buffered saline (PBS) at a density of 5×10^6 cells/ml. Fifty μl of each cell suspension was placed into a PAP Pen (Zymed Laboratories Inc., South San Francisco, CA)-circled area on Superfrost/Plus slide (Fisher Scientific, Pittsburgh, PA) that was coated for cell adhesion. Cells were allowed to adhere to the slide for 10 minutes, washed with PBS and fixed with a 4% paraformadehyde in PBS

solution for 20 minutes. After washing the slides twice with PBS, the cells were permeabilized by treatment with 100 μl of 20 mM SDS in PBS for 10 minutes. Permeabilized cells were washed 3 times with PBS and incubated for one hour at 37°C with a reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein isothiocyanate (FITC)-conjugated digoxigenin-11-UTP for labeling of the exposed 3'-hydroxyl ends of fragmented nuclear DNA. After washing the cells with PBS, a coverslip was mounted onto a slide with PI containing mounting medium (Vector Labs, Bulingame, CA). The fluorescent images of the cells were acquired with a confocal laser scanning microscope (MRC 1024, Bio-Rad, Inc., Richmond, CA). Apoptotic cells having fragmented DNA incorporate abundant amounts of FITC-labeled dUTP and exhibit a green fluorescence. However, non-apoptotic cells incorporate only insignificant amounts of FITC-labeled dUTP due to the lack of exposed 3'-hydroxyl ends in intact DNA and consequently have much less green fluorescence than apoptotic cells. DNA bound PI emits strong red nuclear fluorescence from all the cells.

Results:

As shown in Figs **2** and **3**, exposure to CPI-2 induces apoptosis in the ALL and NHL cell lines (Fig. **2**), but had little effect on inducing apoptosis in the myeloid leukemia cell lines (Fig. **3**). These results correlate with the high expression of calpain in ALL and NHL cells (see Example 1, Fig. 1).

Further, out of the four tested solid tumor cell lines only the solid tumor SQ-20B cells underwent apoptosis when treated with CPI-2 (Fig. **4**). Interestingly, these results closely correlate with the calpain levels expressed in each of the solid tumor cell lines (see Example **2**, Fig. 1).

Apoptosis was confirmed in the ALL, NHL and SQ-20B cell lines using the TUNEL assay.

Taken together, the data shows that CPI-2 selectively induces apoptosis in tumor cell lines that express calpain. At present, ALL and NHL cell lines are suitable targets for calpain inhibitor therapy as well as any cell line, for example SQ20B, that expresses quantities of the calpain protein.

Example IV

Calpain Inhibitor-2 Induced Apoptosis is LYN and BTK-Independent

The materials and methods discussed in Example III were used to determine whether the protein tyrosine kinases LYN or BTK (Uckun et al. (1995) Science, 267: 886-891); Wang et al. (1996) J Exp. Med., 184: 831-838; Vassilev et al. (1996) J Biol. Chem., 274: 1646-1656) were required for CPI-2 induced apoptosis in CPI-2 sensitive cells. Wild type DT-40, LYN deficient and BTK deficient DT-40 lymphoid cells (Uckun et al. (1996) Science, 273: 1096-1100) were examined for susceptibility to CPI-2 induced apoptosis after treatment with 50 μM CPI-2.

Results:

As shown in Figs. **5**, both the wild type and kinase-deficient clones of DT-40 cells were exquisitely sensitive to the cytotoxic activity of CPI-2. This data indicates that neither the tyrosine kinase LYN or BTK are required for CPI-2-induced apoptosis.

Example V

Calpain Inhibitor-2 Induced Apoptosis is Caspase-Dependent

The materials and methods discussed in Example III were used to determine whether the family of caspases (Zuo et al. (1997) Cell, 90: 405-413; Li et al. (1997) Cell, 91: 479-489; Schlegel et al. (1996) J Biol. Chem., 271: 1841-1844; Thornberry et al. (1998) Science, 281: 1312-1316; Green et al. (1998) Science, 281:1309-1312; Henkart (1996) Immunity, 4: 195-201) were required for CPI-2 induced apoptosis in CPI-2 sensitive cells. Cells treated with 50 μM CPI-2 (MOLT-3) or 100 μM CPI-2 (SQ-20B) were incubated with 50 μM caspase inhibitor I (CPI-1) (z-VAD-FMK) (Calbiochem, La Jolla, CA) and tested for apoptosis using the methods discussed above in Example III.

Results:

As shown in Fig. **6**, caspase inhibitor I inhibitited CPI-2-induced apoptosis. This data provides evidence that an apoptosis-promoting caspase system is

activated following calpain inhibition with CPI-2. This data also illustrates a previously unknown cross-talk between the caspase mediators of apoptosis and calpain in neoplastic lymphoid cells and likely cross-talk between the two family members in the normal regulation of cells.

All publications, patents and patent documents described herein are incorporated by reference as if fully set forth. The invention described herein may be modified to include alternative embodiments. All such obvious alternatives are within the spirit and scope of the invention, as claimed below.

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We Claim:

1. A method for inhibiting growth of tumor cells in a subject comprising administering a calpain inhibitor to the subject.

2. The method of claim 1, wherein the inhibitor induces apoptosis of the tumor cells.

3. The method of claim 2, wherein the inhibitor induces caspase dependent apoptosis of the tumor cells.

4. The method of claim 1, wherein the calpain inhibitor is CPI-2.

5. The method of claim 1, wherein the calpain inhibitor is a derivative of CPI-2.

6. The method of claim 1, wherein the tumor cells are selected from the group consisting of acute lymphoblastic leukemia cells and non-Hodgkin's lymphoma cells.

7. The method according to claim 1, wherein the subject has tumor cells with calpain activity.

8. A method of treating cancer cells in a subject comprising administering a therapeutically effective amount of calpain inhibitor.

9. The method of claim 8, wherein the calpain inhibitor is CPI-2.

10. The method of claim 8, wherein the calpain inhibitor is a derivative of CPI-2.

11. The method of claim 8, wherein the cancer is acute lymphoblastic leukemia.

12. The method of claim 8, wherein the cancer is non-Hodgkin's lymphoma.

13. The method of claim 8, wherein the cancer cells have calpain activity.

14. A method for inhibiting inflammatory disease states in a subject comprising administering to the subject a calpain inhibitor.

15. The method of claim 14, wherein the calpain inhibitor is CPI-2.

16. The method of claim 14, wherein the calpain inhibitor is a derivative of CPI-2.

17. The method of claim 14, wherein the inflammatory disease is rheumatoid arthritis.

18. A method for inducing cytotoxicity in a cell comprising:
administering to the cell a cytotoxic dose of a calpain inhibitor.

19. The method of claim 18, wherein the cell is a cancer cell.

20. A pharmaceutical composition comprising a substantially purified calpain inhibitor and a pharmaceutically acceptable carrier.

21. The pharmaceutical composition of claim 20, wherein the calpain inhibitor is CPI-2.

22. The pharmaceutical composition of claim 20, wherein the calpain inhibitor is a derivative of CPI-2.

23. A method of inhibiting the growth of a tumor cell comprising:
administering a calpain inhibitor to the tumor cell.

24. The method according to claim 23, wherein the tumor cell has reduced tyrosine kinase LYN or BTK activity.

25. The method according to claim 24, wherein the tumor cell is LYN deficient or BTK deficient DT-40 lymphoid cells.

26. The method according to claim 23, wherein the tumor cell has calpain activity.

27. A method for inducing apoptosis in cells comprising:
administering a calpain inhibitor to the cells.

28. The method according to claim 27, wherein the cells are tumor cells.

29. The method according to claim 27, wherein the inhibitor is CPI-2.

30. A method for inhibiting growth of tumor cells comprising:
making a recombinant vector that expresses a calpain inhibitor; and
administering the recombinant vector to the tumor cell.

31. The method of claim 30, wherein the recombinant vector expresses CPI-2.

32. The method of claim 30, wherein the recombinant vector expresses a derivative of CPI-2.

33. A method for inducing apoptosis in a cell comprising:

expressing a heterologous nucleic acid sequence encoding a polypeptide that inhibits calpain in a host cell having enhanced calpain activity as compared to control host cells.

FIG. 1

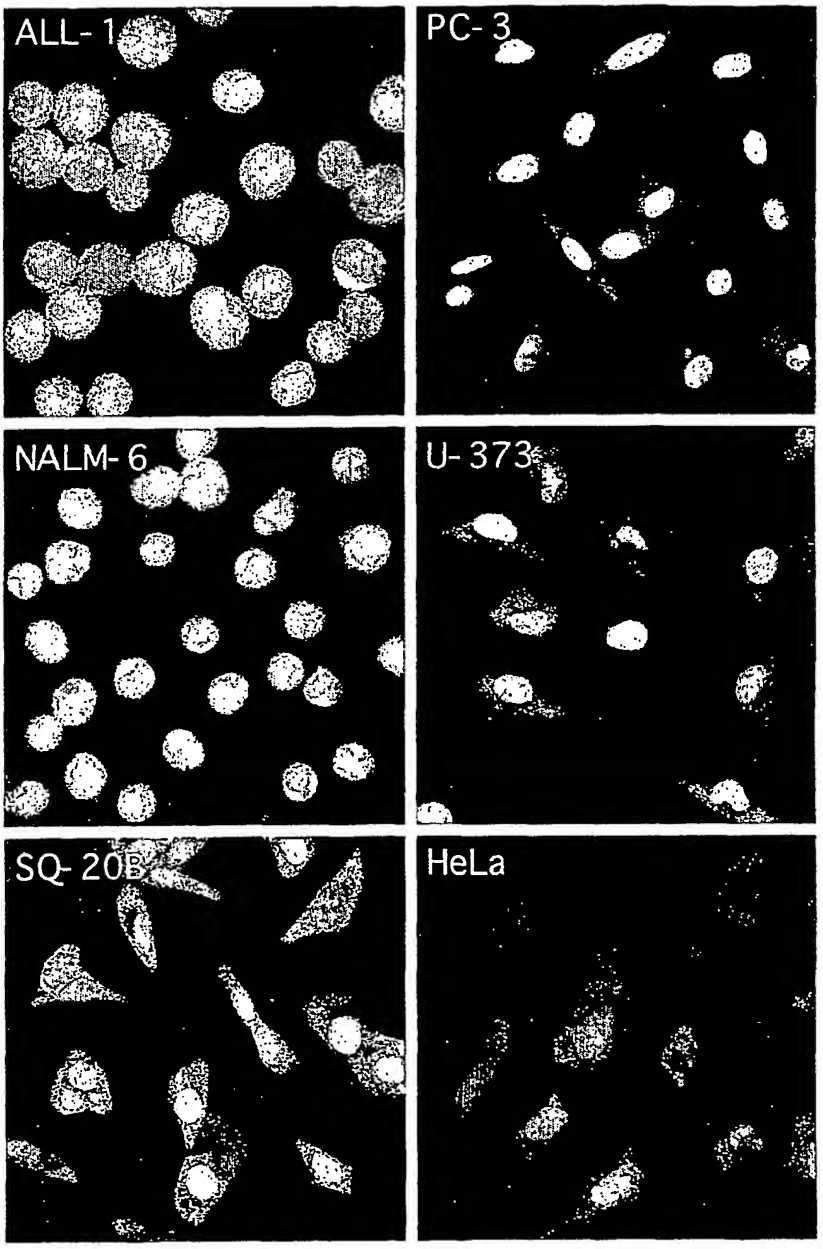


FIG. 2

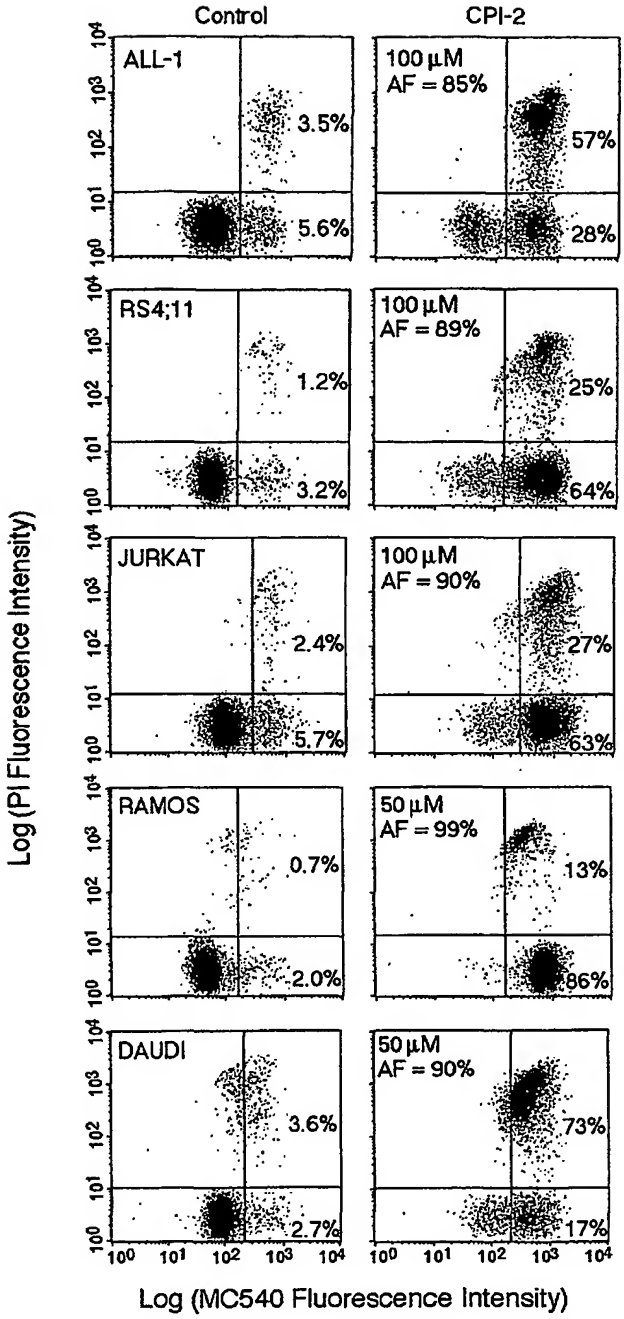

Control
CPI-2
ALL-1
3.5%
5.6%
100 µM
AF = 85%
57%
28%
RS4;11
1.2%
3.2%
100 µM
AF = 89%
25%
64%
JURKAT
2.4%
5.7%
100 µM
AF = 90%
27%
63%
RAMOS
0.7%
2.0%
50 µM
AF = 99%
13%
86%
DAUDI
3.6%
2.7%
50 µM
AF = 90%
73%
17%
Log (PI Fluorescence Intensity)
Log (MC540 Fluorescence Intensity)

FIG. 3

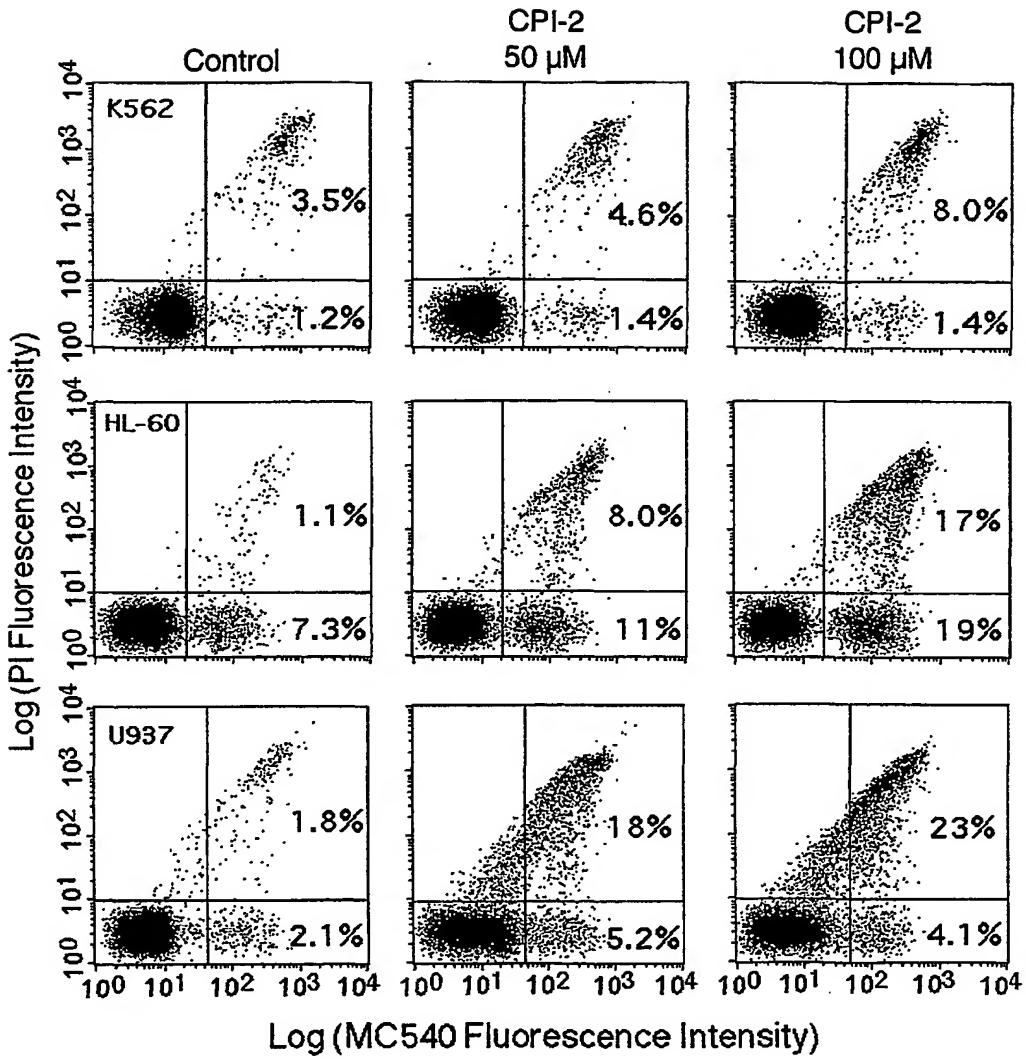

Control
CPI-2
50 µM
CPI-2
100 µM
K562
3.5%
1.2%
4.6%
1.4%
8.0%
1.4%
HL-60
1.1%
7.3%
8.0%
11%
17%
19%
U937
1.8%
2.1%
18%
5.2%
23%
4.1%
Log (PI Fluorescence Intensity)
Log (MC540 Fluorescence Intensity)
10^0 10^1 10^2 10^3 10^4

FIG. 4

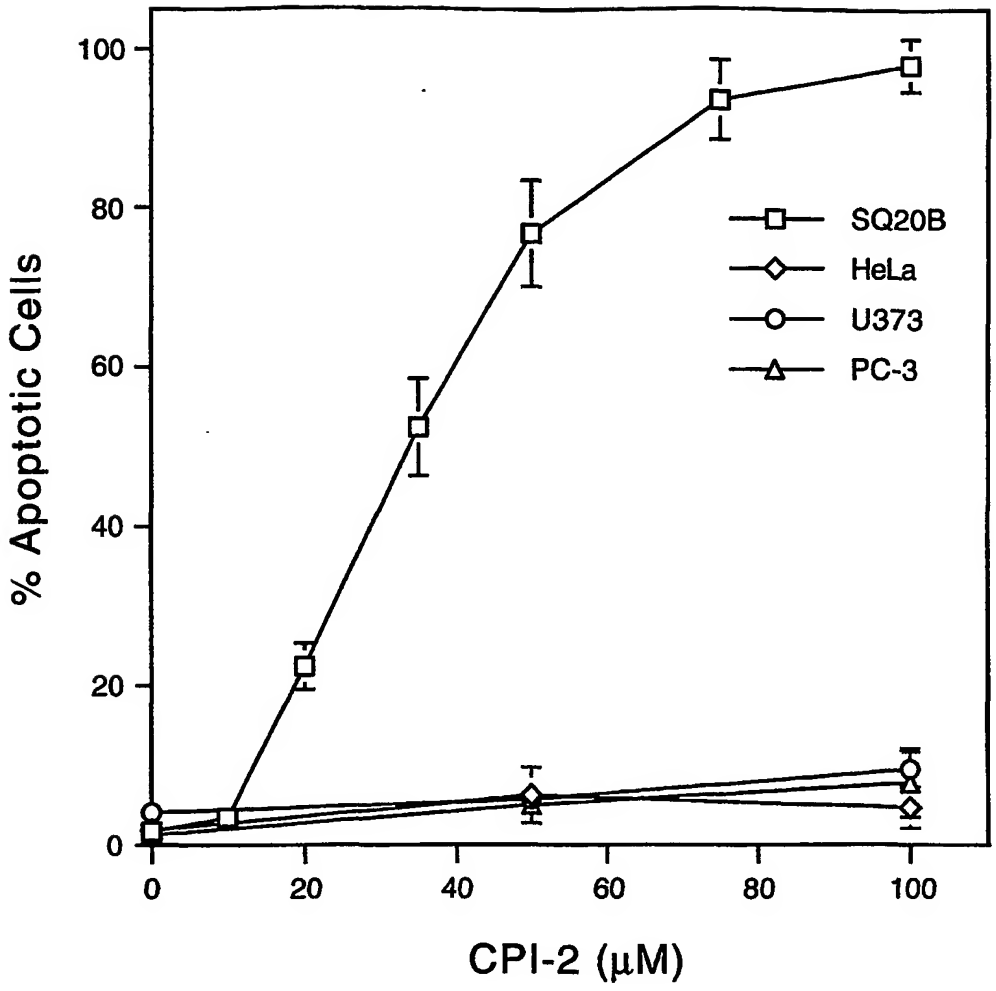

100
80
60
40
20
0
% Apoptotic Cells
0
20
40
60
80
100
CPI-2 (μM)
SQ20B
HeLa
U373
PC-3

FIG. 5

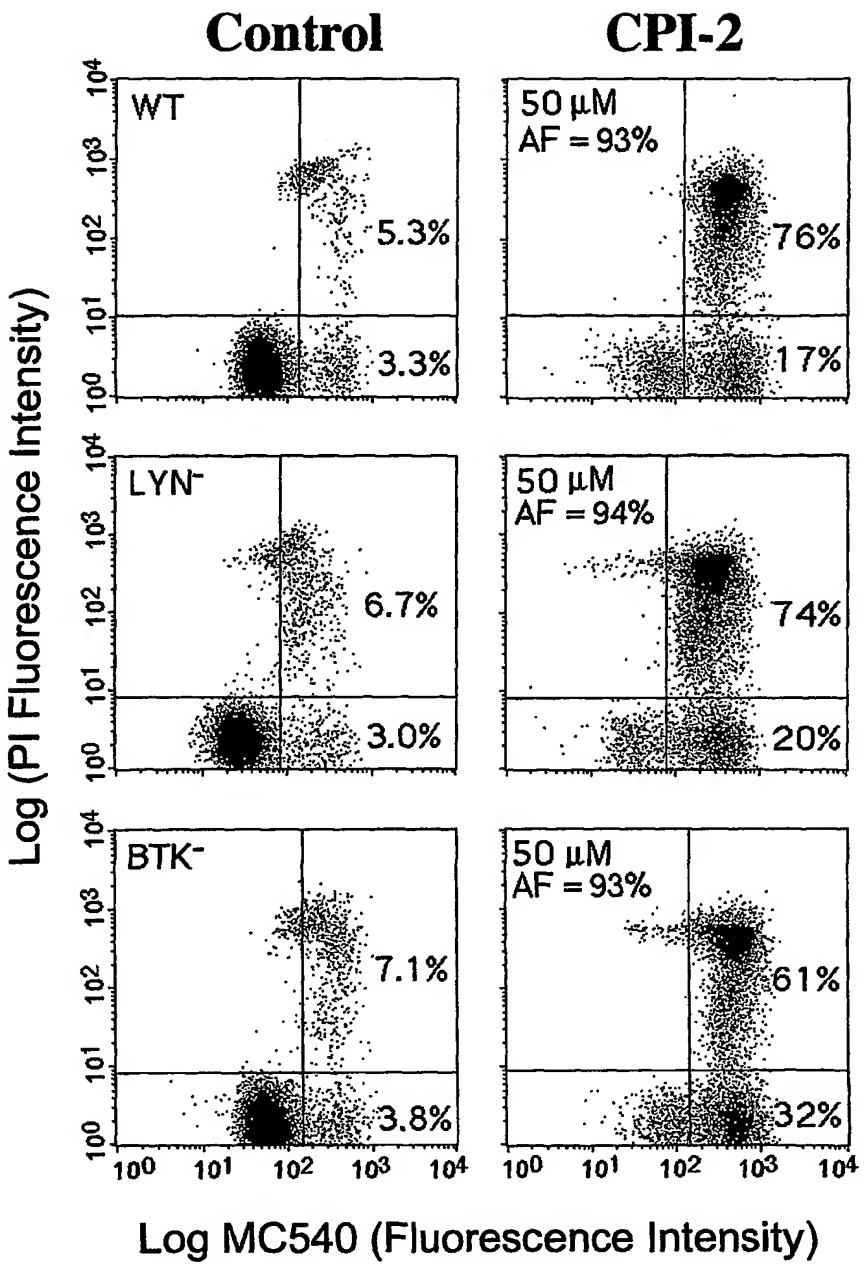
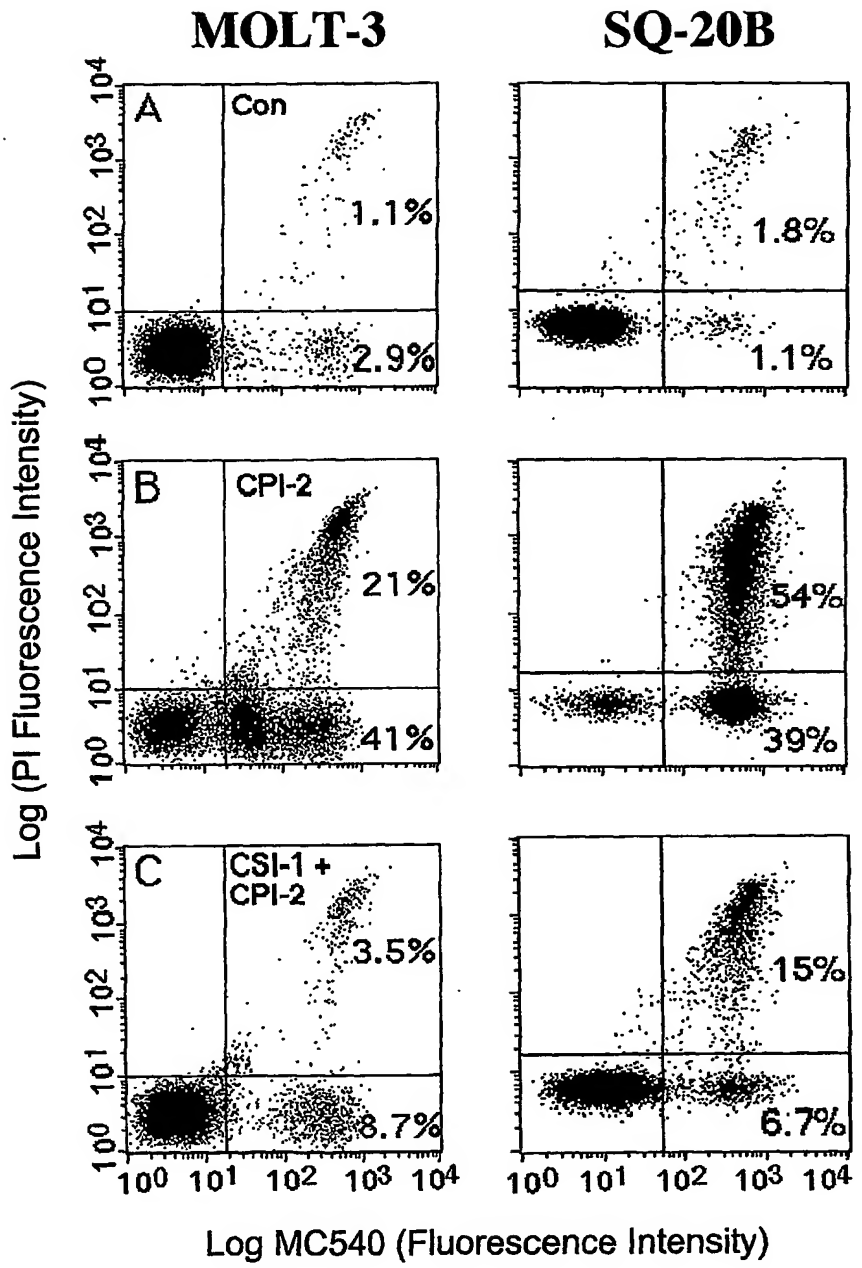

Control
CPI-2
WT
5.3%
3.3%
50 µM
AF = 93%
76%
17%
LYN⁻
6.7%
3.0%
50 µM
AF = 94%
74%
20%
BTK⁻
7.1%
3.8%
50 µM
AF = 93%
61%
32%
Log (PI Fluorescence Intensity)
Log MC540 (Fluorescence Intensity)
10⁰ 10¹ 10² 10³ 10⁴

FIG. 6


MOLT-3
SQ-20B
A
Con
1.1%
2.9%
1.8%
1.1%
B
CPI-2
21%
41%
54%
39%
C
CSI-1 +
CPI-2
3.5%
8.7%
15%
6.7%
Log (PI Fluorescence Intensity)
Log MC540 (Fluorescence Intensity)
10^0 10^1 10^2 10^3 10^4